Attorney Docket No. 5218-39B

PATENT

In re: Anagnostou et al.
Serial No.: 09/525,797
Filed: March 15, 2000
For: *METHOD OF TREATING ENDOTHELIAL INJURY*

Confirmation No. 9917
Group Art Unit: 1642
Examiner: Susan Ungar

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

November 27, 2006

Commissioner for Patents
Post Office Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R § 1.132
OF GEORGE SIGOUNAS, Ph.D.

Sir/Madam:

I, George Sigounas, Ph.D., do hereby declare and say as follows:

1. I received my Ph.D. from Boston University in Cellular Biology. I am currently Professor of Medicine at East Carolina University School of Medicine in Greenville, North Carolina. I am a co-inventor on the above-identified patent application.

2. In view of a telephonic interview on August 29, 2006 with Examiner Ungar and Applicants' representative, Shawna Cannon Lemon, to discuss the issues and rejections raised in the Office Action dated January 27, 2006 and subsequent Advisory Action dated May 3, 2006, the following study designed to evaluate the effect of chemotherapeutic agent mitomycin is discussed below.

3. In this study, the ability of erythropoietin (EPO) to synergize with mitomycin C and inhibit the growth of solid tumors derived from cancerous cells injected into normal animals was assessed. The ability of EPO to modulate the anticancer ability of mitomycin C was tested on Lewis lung carcinoma (LLC) cells-

derived tumors grown in C57BL mice. When these cells are injected subcutaneously, they produce tumors that resist the majority of chemotherapeutic regimens and easily metastasize to the lungs. Mitomycin C alone or in combination with EPO and injected on different time schedules was used. Although EPO alone does not affect tumor growth, we found that EPO may synergize with mitomycin C and further enhance its antineoplastic activity.

To perform the *in vivo* studies, we employed the following protocol.

Methodology

- Cell Cultures

LLC cells were grown in 75 cm^2 T-flasks (Corning Inc, Corning NY) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), supplemented with 2% FBS, 100 u/ml penicillin, and 100 micrograms/ml streptomycin. The cell cultures were incubated at 37°C in a fully humidified environment with 5% CO_2.

- Animals

Female C57BL/6 mice, 7-8 weeks old and obtained from either Harlan Laboratories (Indianapolis, IN) or Jackson Laboratories (Bar Harbor, ME), were used in this study. Mice were housed in plastic cages in groups of 4-8 and allowed ad libitum access to mouse food and water. Animal procedures were approved by the East Carolina University Animal Care Committee.

- Injections

Animals were randomly divided into six treatment groups with 6-16 mice per group. Single cell suspensions of LLC cells (2×10^6/0.1 ml/mouse, >85% viability) were injected subcutaneously into the right front axilla in groups 1-5. The day of cell implantation was designated day 0. After cell injection, tumor growth and tumor appearance were assessed daily. The animals of each group were treated as follows: group 1 (saline), injected with phosphate-buffered saline (PBS); group 2 (EPO), injected with EPO alone; group 3 (MITO), injected with mitomycin C; group 4 (EPO&MITO), injected sequentially first with EPO and then with mitomycin C; group 5 (EPO/MITO), injected simultaneously with EPO and mitomycin C; group 6

(naïve), neither treated nor injected with LLC cells and sacrificed at the end of the study for normal tissue collection. Saline, erythropoietin, and mitomycin C were administered intraperitoneally (i.p.) at a volume of 10μl per gram of body weight. Unless stated otherwise, mitomycin C, EPO alone, and saline were injected on day 6 and day 9 after transplantation of LLC cells. EPO and mitomycin C were used at concentrations of 60 units/mouse and 5 mg/kg, respectively.

- Analysis of Antitumor Activity

Thirteen days following LLC cell injection, animals were euthanized with a high dosage of anesthetic. This time point was selected in order to avoid overgrowth of the tumor. The primary tumors were separated from the surrounding muscles and dermis, excised, weighed and fixed in formalin for histological analysis.

Paired student's t-tests were used to evaluate differences between values. Differences between groups were considered statistically significant at $p<0.05$. Results are expressed as mean values ± SE. Data was analyzed using the Microsoft Excel computer program. The results were obtained from at least four independent experiments.

Results

In these studies, we found that tumor-bearing animals treated with mitomycin C alone had a 2-fold reduction of tumor mass compared to animals injected with saline (see attached Figure). When EPO was injected sequentially, that is, first with EPO and then with mitomycin C, tumor mass was further reduced by 14% compared to that seen in mice treated with mitomycin C alone. Although this difference was not statistically significant, this result indicates that EPO can modulate tumor response to mitomycin C.

Thus, our studies indicate that administering erythropoietin prior to administration of mitomycin can reduce tumor mass *in vivo*, and thus, suggest a protocol employing administration of EPO prior to administration of a chemotherapeutic agent such as mitomycin to treat solid vascularized tumors.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

George Sigounas, Ph.D.

11-28-06
Date